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Poly (ADP-Rib) polymerase requires DNA for activity. It is significant that the catalytic activity of this enzyme is directly coordinated to the number of DNA strand breaks in DNA, both in vitro as well as in vivo. The poly (ADP-Ribosylation) modification of chromatin-associated proteins functions during various biological reactions involving DNA repair and replication). Project aims have been modified during the two years of the course of the project. By utilizing new genetic tools which have been developed in our labatory. We have studied how poly (ADP-ribosylation) helps protect cells from toxic agents which interact with DNA. Specifically, we have been the first group to have reported the cloning of the cDNA and gene for this enzyme. Our aim during the last year was mainly concerned with how to manipulate the hyperexpression of the cloned gene for this enzyme first, to make cells more resistant to toxic agents, and second, to begin to understand the underlying mechanisms by which ADP-Ribosylation alters chromatin around DNA strand breaks to assist cell recovery from such damage.

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THE KEY INVOLVEMENT OF POLY (ADP-RIBSYLATION) IN DEFENSE AGAINST TOXIC AGENTS: MOLECULAR BIOLOGY STUDIES

SUMMARY AND RESEARCH OBJECTIVES:

Poly (ADP-Rib) polymerase requires DNA for activity and it is significant that the catalytic activity of this enzyme is directly coordinated to the number of DNA strand breaks in DNA, both *in vitro* as well as *in vivo*. The poly (ADP-Ribosylation) modification of chromatin-associated proteins thus functions during various biological reactions involving DNA repair, replication).

The project initially had three aims. Some of these aims have been modified during the two years of the course of the project. Most recently, we have been mainly interested in how poly (ADP-ribosylation) helps us to protect cells from toxic agents which interact with DNA, by utilizing new genetic tools which have been developed in our laboratory. Specifically, we have been the first group to have reported the cloning of the cDNA and gene for this enzyme. Our aim during the last years has been mainly concerned with how one might manipulate the hyperexpression of the cloned gene for this enzyme in order to first make cells more resistant to toxic agents and secondly, to begin to understand the underlining mechanism by which ADP-Ribosylation alters chromatin around DNA strand breaks to help the cell to recover from such damage.

STATUS OF THE RESEARCH

Overview of Results and Progress

Progress has been made in a number of related areas concerning the mechanism of the involvement of poly(ADP-ribosylation) in recovery of cells from toxic agents and secondly on the cloning, sequencing chromosomal localization and other aspects of the molecular biology of poly(ADP-Rib) polymerase.

DETAILED REPORT

1. Immunofractionation Characterization of Single Strand DNA Breaks Adjacent to Sites of Poly(ADP-Rib).

Smulson, M., Poly (ADP-Ribosylation) of Nucleosomal Chromatin: Electrophoretic and Immunological Methods, "Methods in Enzymology" 106, 933-943, 1984, Edited by F. Wold Academic Press

Using the method above, we analyzed the nucleosomal populations adjacent and distal to poly (ADP-Rib) for internal single strand breaks by using two-dimensional electrophoretic gels. Relevant to the current project was the observation that **immunofractionated poly (ADP-Rib) oligonucleosomal DNA contains significant amounts of internal single-strand breaks** compared with bulk chromatin.

Overview: This is the first direct data that demonstrates that poly (ADP-ribosylated) nucleosomes are adjacent to strand breaks.

2. Use of Immunofractionation on Anti-Poly (ADP-Rib) Antibody to Study DNA Repair

Thraves, P. J., Kasid, U., and Smulson, M. E., Selective Isolation of Domains of Chromatin Proximal to both the Carcinogen-induced DNA Damage and Poly (ADP-Ribosylation), Cancer Research, 45, 386-391 (1985).

We observed an enhancement (2-fold) in the specific retention of hyper-ADP-ribosylated, in vivo [^{3}H]-TdR labelled chromatin, following treatment with MNU. In addition we noted that the **incorporation** of the chain terminator **Ara-C was also enriched** in the poly (ADP-ribosylated) **strand breaks.** Furthermore, the retention of these chromatin regions to the antibody column was due to the increased synthesis of longer polymer chains on the protein acceptors of these chromatin domains. This methodology thus offers a useful means of isolating the substance under analysis the dynamic domains of chromatin undergoing DNA synthesis and/or repair. Selected data from this paper are shown below.

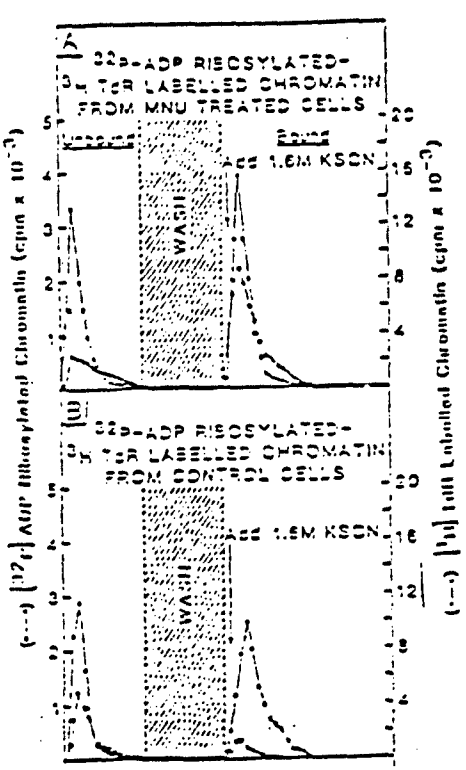


Table 1

Association in vivo of [^{3}H]ara-C with ADP-ribosylated chromatin

HeLa S_3 cells were resuspended in fresh medium at a concentration of 8×10^6/ml and labeled with [^{3}H]ara-C (250 μCi/250 μl) at 8 μCi/ml for 10 min. The cells were then harvested and nuclei purified, and micrococcal nuclease-soluble chromatin were prepared and immunofractionated as described in Chart 1.

Fraction	[^{32}P]Poly(ADP-Rib) (cpm)	[^{3}H]ara-C (cpm)	A_{260nm}[a]	Specific activity of [^{3}H]ara-C
Unfractionated chromatin	1643	3061	4.85	631
Unbound	625 (34)[b]	1903 (62)	4.47 (92)	425
Bound	1218 (66)	1158 (38)	0.38 (8)	3047

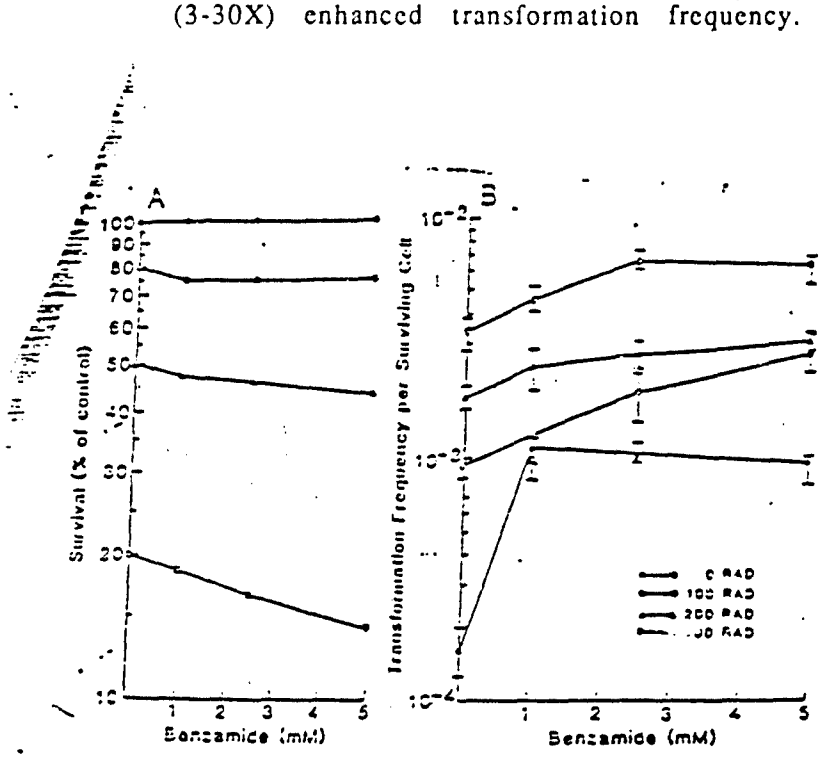
[a] Calculated from a separate unlabeled column run, performed in parallel.
[b] Numbers in parentheses, percentage.

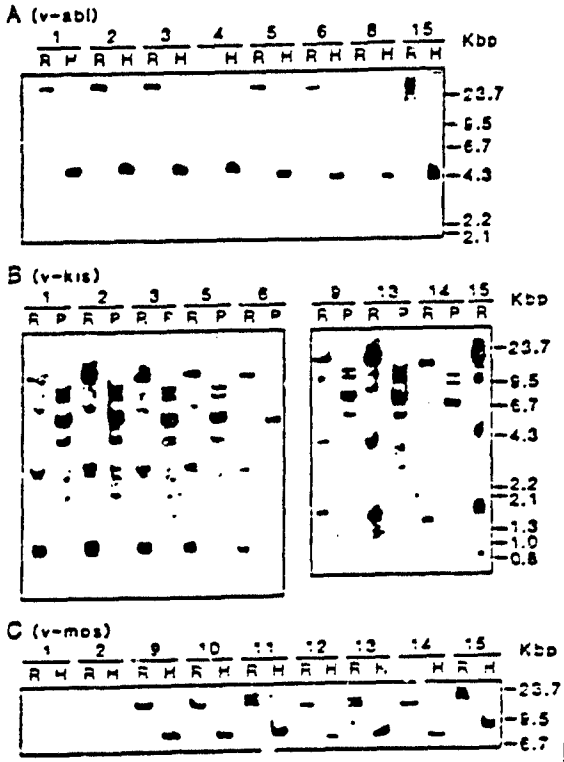
3. Pauses in Poly (ADP-Rib) Subsequent to DNA Strand Breaks Increase 3T3 Cell Transformation, and Oncogene Expression.

Kasid, U. N., Stefanik, D., Lubet, R. A., Dritschilo, A., and Smulson, M. E.: Relationship between DNA Strand Breaks and Inhibition of poly (ADP-ribosylation): Enhancement of Carcinogen-Induced Transformation, Carcinogenesis, 7, 327-330 (1986).

Inhibition of poly (ADP-Rib) of nuclear proteins increases the persistence of DNA strand breaks elicited by DNA damaging agents, and markedly increases SCE exchange reactions. Accordingly, it seemed logical to test, at the molecular level, whether these cellular events cause rearrangements or alterations of specific sequences such as oncogenes in DNA. We utilized transformation of BALB/3T3 cells as a selective system to obtain homogeneous samples of DNA after damaging cellular DNA by X-rays and/or inhibition of poly (ADP-Rib).

Inhibition of poly (ADP-Rib) by benzamide or 3AB for a brief period following DNA damage due to either X-ray or MNNG in BALB/3T3 cells significantly (3-30X) enhanced transformation frequency.





We established 14 transformed cell lines, after having been characterized for growth in soft agar and tumor induction in nude mice. No gross rearrangements of 7 representative oncogenes including the more frequently activated c-ki-ras, was observed (see above, right). DNA dot blot hybridization suggested a 2-4 fold amplification of c-Ha-ras gene in 3 transformed cell lines.

Kasid, U. N., Hough, C., Thraves, P., Dritschilo, A., and Smulson, M.E.; The Association of Human c-Ha-ras Sequences with Chromatin and Nuclear Proteins, Biochem., Biophys. Res. Commuun., 128, 226-232, 1985.

Southern-blot hybridization of DNA isolated form various nucleosomal chromatin fractions to probes of either c-Ha-ras or the inactive B-globin gene yielded the following results: (1) c-Ha-ras sequences segregated predominantly in the nuclease-sensitive chromatin fractions. (2) Unlike the B-globin (inactive gene), C-Ha-ras associated chromatin lacked typical nucleosomal structure. In these preliminary studies we also immuno-fractionated nucleosomes on anti-poly(ADP-Rib) Sepharose to examine the association of this oncogene with sites of poly(ADP-Rib).

Overview: These past studies provide an approach to determine, at the molecular level (i.e. by use of anti-sense), whether inhibitors of poly. (ADP-Rib) polymerase or any other mechanism to reduce DNA repair alone, or in conjunction with damaging agents can cause alterations in oncogene sequences.

4. Poly ADP-Ribosylation of an Oncogene Protein.

Baksi, K., Alkhatib, H., and Smulson, M. E.; *In Vivo* characterization of The Poly ADP-Ribosylation of SV 40 chromatin and Large T Antigen by Immunofractionation, Expt'l. Cell Res., 172, 110-123, 1987.

We have confirmed the poly(ADP-ribosylation) of large T antigen of SV40 by using antibodies to both large T antigen and poly(ADP-ribose) and consequently have begun to characterize how this post-translational nuclear modification of the viral protein modulates its biological functions. SV40 minichromosomal subpopulation containing replicative intermediate DNA was shown to have a significantly higher affinity for anti-poly(ADP-Rib)–Sepharose than viral chromatin fractions containing mature minichromosomal DNA. An anti-large T–Sepharose column was used to isolate T antigen from crude extracts by two different approaches: (1) large T antigen was labeled with [^{35}S]methionine *in vivo* and the infected cell extract was immunofractionated to isolate large T antigen and (2) large T antigen from infected cell extracts was immunofractionated followed by immunostaining. Using these techniques, 1–10% of the total T antigen from infected cells was found to be poly(ADP-ribosylated). Minichromosome preparations *per se* were also subjected to immunofractionation on anti-large T–Sepharose. The high level of retention of poly(ADP-ribosylated) species of minichromosomes on this matrix suggested that this post-translational modification of viral chromatin may be related to those steps in viral replication and transcription under regulation by large T antigen.

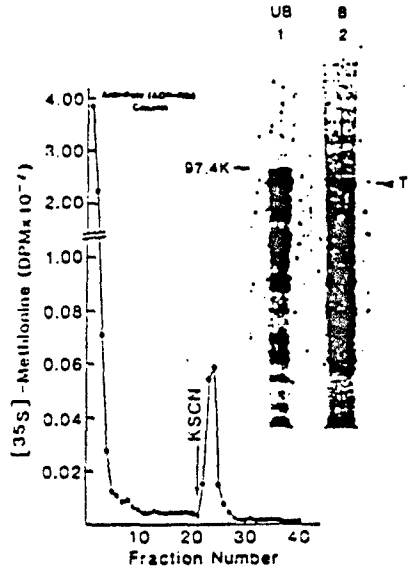


Fig. 3. The natural occurrence of poly(ADP-ribosylation) large T antigen of SV40 virus as determined by immunofractionation. [^{35}S]Methionine-labeled large T antigen isolated on an anti-large T antibody column (Fig. 1) was applied to 6 ml of antipoly(ADP-Rib)–immunoglobulin G–Sepharose 4B as described in Table 1. Acid insoluble [^{35}S]methionine incorporation was monitored in 50 ul of each sample (●). The unbound and bound samples were concentrated and analyzed by SDS–PAGE. The insert shows the autoradiogram of the fractions. Unbound (lane *1*); bound (lane *2*).

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Vol. 84, pp. 1224–1228, March 1987
Biochemistry

Cloning and expression of cDNA for human poly(ADP-ribose) polymerase

(DNA repair/COS cell transfection/cell cycle/DNA strand breaks)

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Communicated by Arthur B. Pardee, November 10, 1986

ABSTRACT cDNAs encoding poly(ADP-ribose) polymerase from a human hepatoma λgt11 cDNA library were isolated by immunological screening. One insert of 1.3 kilobases (kb) consistently hybridized on RNA gel blots to an mRNA species of 3.6–3.7 kb, which is consistent with the size of RNA necessary to code for the polymerase protein (116 kDa). This insert was subsequently used in both *in vitro* hybrid selection and hybrid-arrested translation studies. An mRNA species from HeLa cells of 3.6–3.7 kb was selected that was translated into a 116-kDa protein, which was selectively immunoprecipitated with anti-poly(ADP-ribose) polymerase. To confirm that the 1.3-kb insert from λgt11 encodes for poly(ADP-ribose) polymerase, the insert was used to screen a 3- to 4-kb subset of a transformed human fibroblast cDNA library in the Okayama–Berg vector. One of these vectors [pcD-p(ADPR)P; 3.6 kb] was tested in transient transfection experiments in COS cells. This cDNA insert contained the complete coding sequence for polymerase as indicated by the following criteria: (*i*) A 3-fold increase in *in vitro* activity was noted in extracts from transfected cells compared to mock or pSV2-CAT transfected cells. (*ii*) A 6-fold increase in polymerase activity in pcD-p(ADPR)P transfected cell extracts compared to controls was observed by "activity gel" analysis on gels of electrophoretically separated proteins at 116 kDa. (*iii*) A 10- to 15-fold increase in newly synthesized polymerase was detected by immunoprecipitation of labeled transfected cell extracts. Using pcD-p(ADPR)P as probe, it was observed that the level of poly(ADP-ribose) polymerase mRNA was elevated at 5 and 7 hr of S phase of the HeLa cell cycle, but was unaltered when artificial DNA strand breaks are introduced in HeLa cells by alkylating agents.

In the above work we showed that the full-length cDNA for poly(ADP-Rib) polymerase, in an appropriate expression vector, is capable of causing hyperexpression of this enzymatic activity in cells already possessing endogenous activity for the enzyme. For example, in the figure from this paper shown below, cells were transfected with the polymerase cDNA under transient transfection conditions and 48 hrs after the initial transfection the cells were isolated and analyzed for both activity and also concentration of poly(ADP-Rib) polymerase caused by the cloned cDNA.

In Panel B in the figure below the enzymatic activity of polymerase was assayed in extracts from Cos cells as well as other cells which had been transfected with control plasmids or mock transfected. In Lane 4 it is obvious that greatly enhanced enzymatic potential for poly ADP-ribosylation existed due to the cloned gene. This was followed up by immunoprecipitating the newly synthesized polymerase due to the cloned gene in the various extracts. Again, it is obvious in Lane 9 that considerably increased amounts of poly(ADP-Rib) polymerase are synthesized in these cells due to the expression vector.

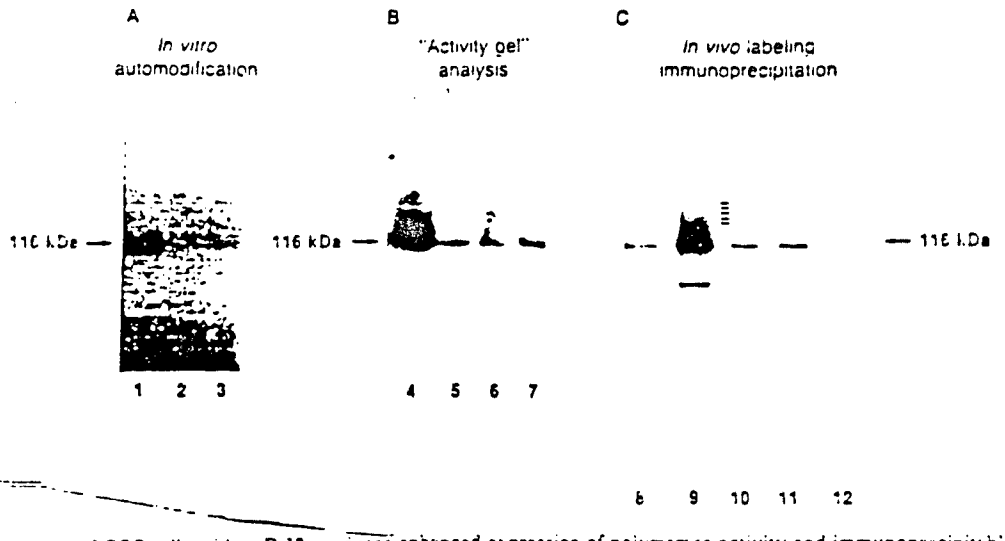

FIG. 5. Transfection of COS cells with pcD-12 produces enhanced expression of polymerase activity and immunoprecipitable polymerase protein. COS cells (2×10^6 cells) in duplicate flasks were treated in the presence or absence of plasmid DNA (25 μg per 175-mm flask) for 4 hr at 37°C in the presence of DEAE-dextran as described (25). After 48 hr, the cells were rinsed, scraped from plates, sonicated, and assayed for

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Biochemistry

cDNA sequence, protein structure, and chromosomal location of the human gene for poly(ADP-ribose) polymerase

(DNA binding protein/DNA-strand-break repair/chromosomes 1, 13, and 14/restriction-fragment-length polymorphism)

BARRY W. CHERNEY*, O. WESLEY MCBRIDE†, DEFENG CHEN*, HUSSEIN ALKHATIB*, KISHOR BHATIA*, PRESTON HENSLEY*, AND MARK E. SMULSON*‡

*Department of Biochemistry, Georgetown University Schools of Medicine and Dentistry, Washington, DC 20007, and †Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Communicated by Roscoe Brady, August 18, 1987 (received for review July 1, 1987)

ABSTRACT **Recently we described a full-length cDNA for the human nuclear enzyme poly(ADP-ribose) polymerase. Here, we report the chromosomal localization and partial map of the human gene for this enzyme as well as the complete coding sequence for this protein. The nucleotide sequence reveals a single 3042-base open reading frame encoding a protein with a predicted M_r of 113,135. A comparison of this deduced amino acid sequence with the amino acid sequence of three peptides derived from human poly(ADP-ribose) polymerase revealed a match of 27 amino acid residues. A computer-derived structural analysis of the enzyme and a search for similarities with other proteins confirmed that the polymerase belongs to a subfamily of DNA/NAD-binding proteins and DNA-repair proteins. Possible Zn^{2+}-binding "fingers," a nucleotide-binding fold, and a nuclear transport signal were noted. Additionally, chromosomal mapping has identified polymerase-hybridizing sequences on human chromosomes 1 (the active gene), 13, and 14 (processed pseudogenes). Using the polymerase cDNA as a probe, we also have detected several DNA restriction fragment length polymorphisms in normal humans.**

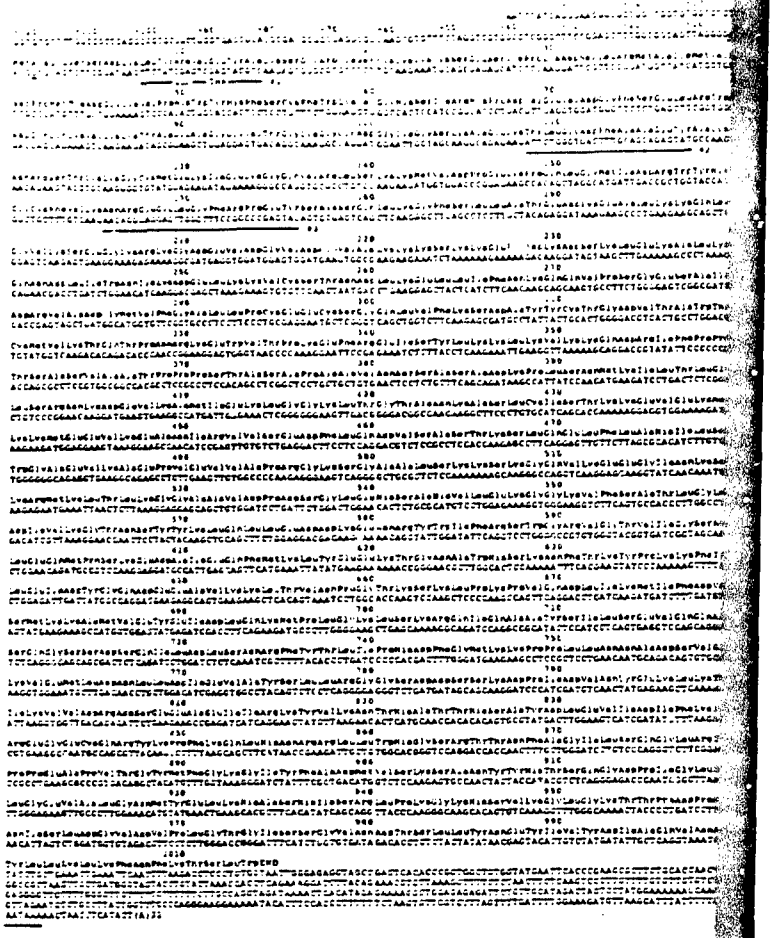


FIG. 2. Nucleotide sequence of the Okayama–Berg pCD-poly(ADP-ribose) polymerase cDNA insert and the deduced amino acid sequence of the 113,135-Da protein. The protein contains sequences coding for three poly(ADP-ribose) polymerase peptides (underlined and sequenced), in the 3′ untranslated region, a putative mRNA processing signal (AATAAA) is underlined. In the 5′ region two nucleotides corresponding to the Kozak criteria for initiation are underlined (25).

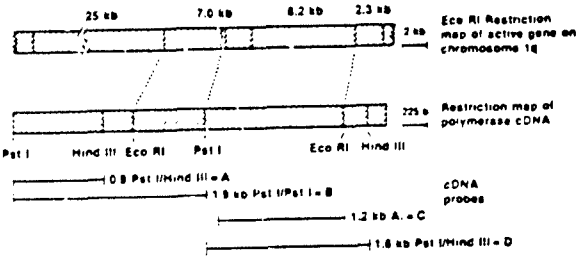


FIG. 5. Genomic organization of poly(ADP-ribose) polymerase. The alignment of similar *Eco*RI sites present in the genomic and cDNA maps is illustrated together with cDNA probes used to order the *Eco*RI fragments. The approximate location of the *Pst* I cDNA site is also shown. (Separate scales have been used to construct the genomic and cDNA maps.) b, Base.

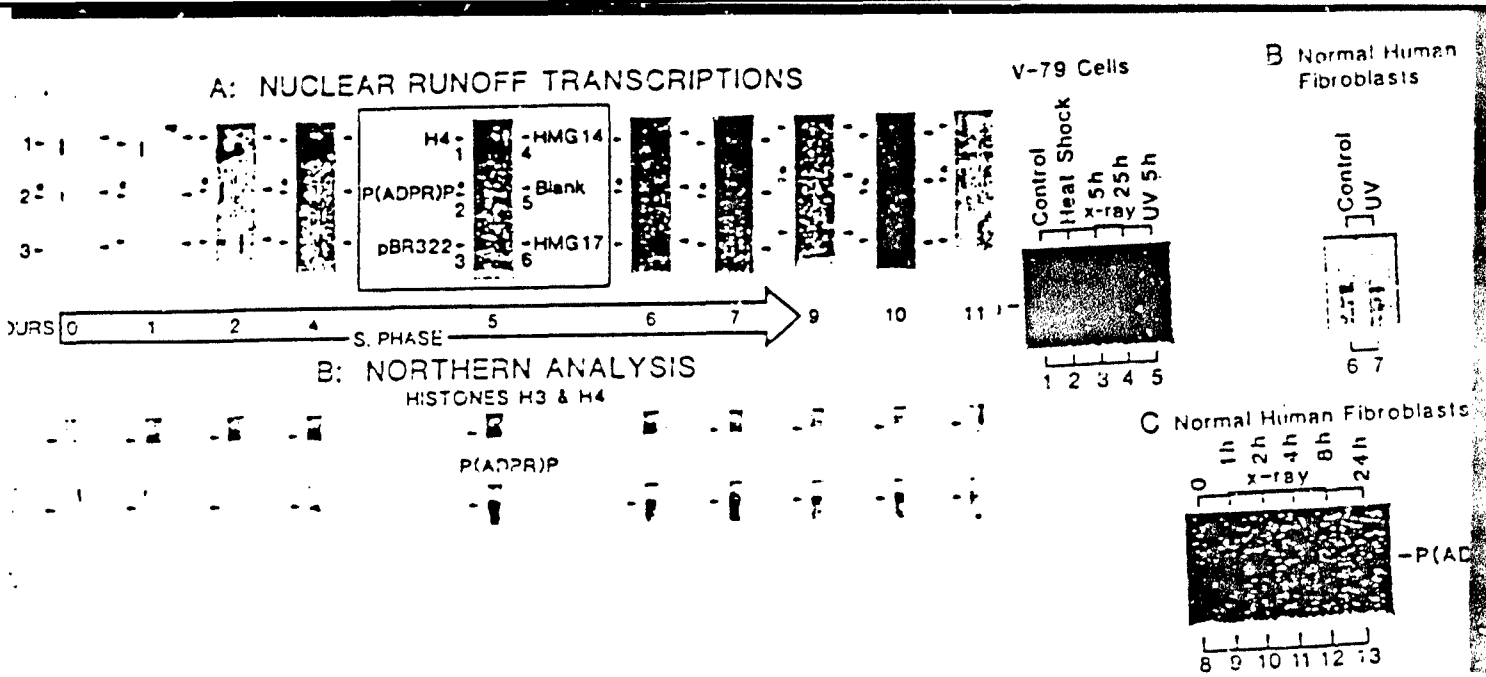
Overview: These two studies (as supported by this as well as other sources) have culminated a three year program on the total sequencing of the cDNA for poly(ADP-Rib) polymerase as well as a formal study on the chromosome localization of the polymerase gene. We are thus now able to analyze the various functional domains (i.e., active site, DNA binding domain, etc.) of the protein. We plan to use this information to engineer important regions of the enzyme into various expression vectors such to manipulate, in cells, the various biological functions of poly(ADP-Rib) polymerase. Additionally, site-directed mutants will be useful for new experiments. The chromosomal localization of polymerase gene has allowed us to assign restriction sites to isolate the total active gene for the enzyme. The isolation of the gene may be very useful in future years in order to develop cells containing not only the cDNA sequences but also active gene sequences including introns.

6. Preliminary Progress on Regulation of Expression of Polymerase Gene After DNA Strand Breakage.

Bhatia, K., Stein, G. S., Bustin, M., Fornace, A. J., Imaizumi, M., Breitman, T. R., Cherney, B. W., and Smulson, M. E.; Expression of The Poly(ADP-Ribose) Polymerase Gene: The Cell Cycle, Differentiation, and DNA Repair (Submitted), J. Biol. Chem, 1987

Having isolated the full-length cDNA for the polymerase, we have now evaluated the role of endogenously and exogenously induced DNA strand breaks on the transcriptional control of this enzyme. During cell replication and differentiation significant changes were found in mRNA levels for the polymerase. In a synchronized population of HeLa cells or in serum-stimulated WI-38 cells, steady state levels of the polymerase mRNA were highest at mid S and S-G_2 phases and negligible in early S phase. Transcription by *in vitro* nuclear run-off, showed a 4-fold increased level of newly synthesized RNA occurring in mid S phase. Unlike histones, the polymerase run-off transcription was independent of the continued replication of DNA during S phase. Similar to a number of growth related gene transcripts that are selectively degraded, the polymerase mRNA has an AUUUA motif in the 3' untranslated region. We conclude from this that mRNA pools for the polymerase are regulated both at the transcriptional and post-transcriptional levels.

During retinoic acid or DMSO-induced differentiation of HL 60 cells mRNA levels for the polymerase increased very early and remained high for up to 48 hours following which it decreased to pre-induced levels. None of these changes, however, were found to accompany the induction of exogenously caused DNA strand breaks as occasioned by either dimethylsulfate, X-irradiation, or UV-irradiation. It appears that in sharp contrast to the catalytic requirement of the polymerase, the induction of transcription of this gene may not be a strand break-dependent process.



7. Hyper-Expression of Polymerase Causes Increased DNA Repair.

Bhatia, K., Giri, C., Pommier, Y., Chemey, B., Dritschilo, A., Alkhatib, H., and Smulson, M.; Enhanced rate of DNA strand break repair by hyper expression of recombinant human poly(ADP-Rib) polymerase. (Submitted), 1988.

ABSTRACT

The nuclear enzyme poly(ADP-ribose) polymerase has been implicated in mediating the repair process of mammalian cells exposed to various DNA insults. Studies of poly ADP-ribosylation *in vivo* have also supported a mechanism by which the generation of DNA strand breaks is a requirement to activate the enzyme. Inhibitors of the poly ADP-ribosylation reaction cause a reduction in the rejoining of DNA strand breaks in cells after DNA damage, the potentiation of the cytotoxicity of alkylating agents, an increase in the extent of sister chromatid exchange reactions as well as ionizing radiation induced cell transformation. Recently we described the first cloning of poly(ADP-ribose) polymerase cDNA from a human gt-11 library. A 3.7-kb full length cDNA contained in the Okayama-Berg expression vector was subsequently isolated and tested for transient expression in Cos cells. Results indicate that a 3 fold increase in specific activity of poly(ADP-ribose) polymerase is present over endogenous levels in transfected cells. The high level of expression thus provided for the first time a direct test for the hypothesis of an involvement of poly ADP ribosylation in DNA repair by a positive enhancement of enzyme levels rather than indirectly by the use only of inhibitors. Cos cells allowed to overexpress the polymerase were X-irradiated and subsequently allowed to repair. Residual single strand breaks in transfected cells were significantly lower at 15 mins following repair as compared to single strand breaks in mock transfected or non transfected cells. When Cos cells were transfected with plasmid containing a nonfunctional polymerase cDNA the enhanced repair capacity was abolished. Increased repair capacities of overexpressing Cos cells were also compromised in the presence of 3-aminobenzamide.

PUBLICATIONS DURING THE LAST 2 1/2 YEARS (11/85-2/88)
From this and other support in the laboratory

1. FULL PAPERS:

Thraves, P. J., Kasid, U., and **Smulson, M. E.**; Selective Isolation of Domains of Chromatin Proximal to both Carcinogen-Induced DNA Damage and Poly(Adenosine-Diphosphate-Ribosylation), Cancer Research, 45, 386-391 (1985).

Kasid, U. N., Hough, C., Thraves, P., Dritschilo, A., and **Smulson, M. E.**; The Association of Human c-Ha-ras Sequences with Chromatin and Nuclear Proteins, Biochem. Biophys. Res.Commun., 128, 226-232 (1985).

Kasid, U. N., Stefanik, D., Lubet, R. A., Dritschilo, A., and **Smulson, M. E.**; Relationship between DNA Strand Breaks and Inhibition of poly (ADP-ribosylation): Enhancement of Carcinogen-Induced Transformation, Carcinogenesis, 7, 327-330 (1986).

Baksi, K., Alkhatib, H., and **Smulson, M. E.**; *In Vivo* characterization of The Poly ADP-Ribosylation of SV 40 chromatin and Large T Antigen By Immunofractionation, Expt'l. Cell Res., 172, 110-123 (1987).

Alkhatib, H., Chen, D., Cherney, B., Bhatia, K., Notario, B., Slattery, E., Giri, C., Stein, G., Roeder, R., and **Smulson, M.**; Cloning and Expression of cDNA for Human Poly(ADP-Rib) Polymerase, Proc. Natl. Acad. Sci., 84, 1224-1228 (1987).

Cherney, B., McBride, O. W., Chen, D., Alkhatib, H., and **Smulson, M.**; DNA Sequence, Protein Structure and the Chromosomal Location of the Human Gene for Poly(ADP-Rib) Polymerase, Proc. Natl. Acad. Sci. USA, 84, 8370-8374 (1987).

PAPERS SUBMITTED:

Kasid, U. N., Prieto-Soto, A., Halligan, B., Liu L. F., Dritschilo, A., and Smulson, M. E.; Poly(ADP-ribosylation) of chromatin associated human topoisomerase I: *In Vitro* and *in vivo* analysis. (Submitted), 1988.

Bhatia, K., Giri, C., Pommier, Y., Chemey, B., Dritschilo, A., Alkhatib, H., and Smulson, M.; Enhanced rate of DNA strand break repair by hyper expression of recombinant human poly(ADP-Rib) polymerase. (Submitted), 1988.

Bhatia, K., Stein, G. S., Bustin, M., Fornace, A. J., Imaizumi, M., Breitman, T. R., Chemey, B. W., and Smulson, M. E.; Expression of The Poly(ADP-Ribose) Polymerase Gene: The Cell Cycle, Differentiation, and DNA Repair. (Submitted), 1988.

RECENT CHAPTERS IN BOOKS:

Smulson, M. Poly (ADP-Ribosylation) of Nucleosomal Chromatin: Electrophoretic and Immunological methods, "Methods in Enzymology 106, 933-943 (1984), Edited by F. Wold. Academic Press, New York.

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